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SEARCH FOR HEPATITIS A VIRUSES BY NEW METHODS

Annual Progress Report

Joseph L. Melnick, Ph.D.

September 1981

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DD Form 1473, Item 20: Abstract

A preparation of HBsAg/ayw vaccine, which has been previously characterized in chimpanzees, was set aside by Dr. J. Gerin in cooperation with the National Institute of Allergy and Infectious Diseases for characterization of an HBsAg-derived polypeptide vaccine. The major polypeptides (mol. wt. 22,000 and 25,000) were derived by SDS-polyacrylamide gel electrophoresis (PAGE) and pooled. This was designated as a P22 polypeptide vaccine. The immunogenicity of freshly prepared, as well as material stored at 4° and 20°C for a period of 6 months, was tested in mice. Consistent antibody activity was noted in mice immunized with P22, but the immunogenicity was markedly lower than that noted in mice inoculated with the intact particle vaccine. Inoculation of P22 vaccine in conjunction with tetanus toxoid significantly increased the immunogenicity of the P22 component when saline suspensions were compared. In contrast, alum-adsorbed preparations showed a decline in the geometric mean titer when tetanus toxoid was added.

Recent studies ~~by this laboratory~~ have shown that patients who have recovered from HBV infections develop a specific cellular cytotoxicity toward the PLC/PRF/5 hepatoma cell line. These observations were extended by analysis of possible specific cellular cytotoxicity in three persons passively immunized with anti-HBs globulin and in 20 individuals vaccinated with HBsAg vaccine. The three subjects with passively administered antibody activity were negative for cellular activity, but in 2 of 20 individuals vaccinated with formalinized HBsAg vaccine, a specific cytotoxicity for hepatoma target cells was noted.

To facilitate testing of large numbers of mouse antisera, a micro solid-phase radioimmunometric assay (micro-SPRIA) and an enzyme-linked immunosorbent assay (ELISA) were developed and compared to the commercial radioimmunoassay (Ausab) for detection of anti-HBs antibody. The two solid-phase antibody assays proved to be 200-350X more sensitive for detection of antibody than the commercial antibody assay kit.

Preliminary data were presented in conjunction with an undenatured P22-P25 polypeptide vaccine prepared by the technology of Zuckerman and coworkers. The polypeptide material, isolated in micelle form, retained high levels of HBsAg activity when tested by solid-phase radioimmunoassay in contrast to the low activity observed with SDS-denatured material. This material has been inoculated into mice to test its relative level of immunogenicity.

HAV has been purified from both marmoset liver tissue and from chimpanzee fecal material. Four weeks after inoculation of PLC/PRF/5, Mahlavu and MC-5 cells a significant increase of HAV antigen was noted in the culture media by RIA procedures.

A waterborne outbreak of gastroenteritis and hepatitis occurred in Georgetown, Texas, in June 1980. Along with human enteric viruses, HAV was detected in the sewage and in one well water sample by micro-SPRIA procedures.

Foreword:

In conducting the research described in this report, the investigator(s) adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences-National Research Council.

TABLE OF CONTENTS

I. Immunogenic Characterization of the NIAID Stock of HBsAg/ayw Vaccine to be Used as a Source for a Possible Polypeptide Vaccine

Dr. John Gerin, in cooperation with the National Institute of Allergy and Infectious Diseases (NIAID), has set aside 60 mg of a purified preparation of HBsAg/ayw to be utilized as a possible source of material for a polypeptide vaccine suitable for human inoculation. This will be referred to as the NIAID vaccine. Since the starting material was safety tested in chimpanzees and was found to contain no residual infectivity, it was assumed that a polypeptide pool derived from this material could forego further testing in chimpanzees.

Preparation of the 22-nm particle antigen was carried out as previously described (1). The basic characteristics of this vaccine have been described (2). The purified preparations were inactivated by the addition of formalin at a 1:2000 dilution and were incubated at 37°C for 96 hr. Both antigenicity and efficacy studies were carried out in chimpanzees. Subsequent to the above studies, we have tested the immunogenicity of the intact particle vaccine and of a derived P22-P25 polypeptide vaccine in mice.

The first series of experiments was designed to test the immunogenicity of the intact particles both as an aqueous preparation and as an alum precipitate. Alum precipitation was performed as described in previous reports. A group of 6 mice was injected with the alum-precipitated preparation and 5 mice were immunized with an aqueous suspension. Each mouse was inoculated by the intraperitoneal route with 10 µg HBsAg in a total volume of 200 µl. The postinoculation days of tail vein bleedings and the time of the single booster inoculation are shown in Table 1. The data are expressed as P/N values determined by testing 0.2 ml of a 1:4 serum dilution using a commercial Ausab kit (Abbott Laboratories, North Chicago, Ill.). As noted in Table 1, a vigorous antibody response was seen 13 days after the primary inoculation of alum-precipitated material (P/N value range from 18 to 45) which remained essentially unchanged or increased on days 31 and 73. A large elevation of antibody activity was noted in all 6 mice 17 days after a booster inoculation of alum-precipitated vaccine. Although the titers were significantly lower in those mice injected with the aqueous preparation, all 5 mice did have a positive response by day 13. In 4 of these 5 mice, detectable levels of antibody were also noted on days 31 and 73. The antibody response noted after a booster inoculation of aqueous vaccine was significant, with P/N values ranging from 25 to 265.

Our next experiments were designed to test the immunogenicity of a P22-P25 polypeptide preparation derived from the NIAID vaccine by the procedures described above. The purity of our polypeptide pool was monitored by analytical slab PAGE (polyacrylamide gel electrophoresis), and only the P22 and P25 bands were noted. The preparations were adjusted to the same protein concentration used above (10 µg protein/200 µl). The material was precipitated with alum. It was estimated that the original NIAID vaccine contained a residual level of formalin (approximately 1:20,000). Therefore, one aliquot of the alum-precipitated polypeptide was treated with formalin to give a final concentration of 1:20,000. The antibody responses noted after inoculation of mice are shown in Table 2. Several observations can be made from this experiment. First, essentially all immunogenic activity was destroyed by treatment of the HBsAg polypeptide preparation with 1:20,000 formalin. Only 1 of 6 mice responded with a very weak antibody response. Second, 6 of 6 mice produced detectable levels of antibody at days 7 and 14 after the primary inoculation of untreated polypeptide vaccine. Unfortunately, formalin had been added to the major portion of our polypeptide preparation so both groups of mice were injected with a second inoculation containing formalin-treated material. No significant response was noted (Table 2).

Another question that was addressed during the current contract year dealt with the stability of the alum-precipitated polypeptide vaccine after storage at 4°C. In earlier studies (3, 4), we noted an apparent loss of immunogenicity when a similar preparation was held at 4°C for a period of 3 months. Therefore, we prepared a new alum-precipitated, NIAID-derived polypeptide vaccine that had not been treated with formalin. This material was used for animal inoculation after storage at 4°C for 2 months. The antibody responses (Table 3) were similar to those observed when mice were injected with an untreated polypeptide vaccine prepared freshly (Table 2). Again, 4 of 6 animals displayed antibody activity 72 days after the primary inoculation, but the level of activity was quite low.

The stability of this antigen will be retested for immunogenicity after storage of two alum-precipitated polypeptide preparations for a period of 6 months at 4 and -20°C, respectively.

II. Inoculation of a Particle HBsAg or an HBsAg Polypeptide Vaccine in Conjunction with Tetanus Toxoid

Purification of 22-nm HBsAg particles was done as previously described (5, 6).

The HBsAg-derived P22-P25 polypeptide pool was obtained by preparative PAGE as previously described (7). This polypeptide pool will be designated as a P22 polypeptide vaccine in the remainder of this report. Purified HBsAg/*adw* particles were disrupted by heating at 100°C for 2 min in the presence of 0.5 M urea, 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol (all from Bio-Rad Laboratories, Richmond, Calif.). PAGE was run on a 10% polyacrylamide gel (Bio-Rad Laboratories). The protein bands were localized on a small wedge-shaped section cut longitudinally from the gel and were stained with 0.25% Coomassie brilliant blue. The segment from the unstained gel, corresponding to P22, was cut out and homogenized. The protein was then eluted from the gel, concentrated by lyophilization, and checked for purity by analytical slab gel electrophoresis (8).

Tetanus toxoid (TT) (Connaught Laboratories, Inc., Swiftwater, Pa.) was purchased as a solution of fluid toxoid in isotonic sodium chloride containing approximately 8 Lf/50 µg protein/ml.

Four different types of preparations were used for each antigen: (i) 10 µg protein suspended in 300 µl 0.9% sterile saline solution (HBs-saline, P22-saline, TT-saline); (ii) 10 µg alum-precipitated protein suspended in 300 µl phosphate-buffered saline (PBS) (HBs-alum, P22-alum, TT-alum); (iii) 10 µg viral protein and 10 µg TT mixed in 0.9% sterile saline solution to a final volume of 300 µl (HBs+TT-saline, P22+TT-saline); (iv) 10 µg viral protein and 10 µg TT, coprecipitated on alum gel, suspended in 300 µl PBS (HBs+TT-alum; P22+TT-alum). Groups of 6 adult BALB/c mice were inoculated intraperitoneally with each designated vaccine preparation, then boostered twice at 2-week intervals. Each inoculum consisted of the above-mentioned doses of protein or protein mixture. The mice were evaluated for humoral immune response 10 days after the second booster.

Alum-precipitated preparations were prepared by adsorbing the proteins to a gel of aluminum potassium sulfate [$AlK(SO_4)_2 \cdot 12H_2O$; Fisher Scientific Co., Fair Lawn, N.J.] as described previously (3). Gel preparations with a single protein (HBs, P22 or TT), as well as with mixed proteins (HBs+TT and P22+TT), were obtained. A 10% (0.21 M) solution of hydrated aluminum potassium sulfate was prepared in 0.01 M PBS, pH 6.2. The necessary amount of 10% alum salt was calculated according to the amount of protein to

be bound, to assure a proportion of 0.8 µg aluminum ion (Al^{3+}) for 100 µg protein. The solution of protein(s) and the 10% aluminum salt solution were mixed to a final volume of 10 ml with 0.01 M PBS, pH 6.2. The pH was adjusted to 5 with 1 N NaOH, and adsorption proceeded for 2 hr at room temperature with gentle stirring. The gel was then washed twice with 0.9% saline solution and resuspended in 0.05 M PBS, pH 7.2, to a final concentration of 10 µg protein in 300 µl for individual preparations and to 10 µg of each protein in 300 µl for mixed preparations.

The efficiency of adsorption of each individual protein onto the washed gels was determined by monitoring the cpm of the trace labeled antigen at each step of the procedure. The final washed gel preparations were suspended to a concentration of 10 µg protein/300 µl based on the adsorption efficiency. The amount of HBs, P22, and TT bound in individual preparations was in excess of 95% (3). When HBs or P22 were coprecipitated with TT on the same gel, the percent of adsorption was lowered for each protein to approximately 70-75%.

The iodination of HBsAg particles as well as proteins (P22, TT) has been described (9, 10). Over 90% of the radioactivity was precipitated with 20% trichloroacetic acid. The specific activities of labeled preparations ranged from 3 to 5 µCi/µg for HBs, from 8 to 16 for P22 (10), and from 7 to 15 for TT.

Mice were immunized against purified 22-nm HBsAg/*adw* particles (subunit vaccine), HBsAg/*adw*-derived P22 polypeptide (polypeptide vaccine), and TT, in individual or combined vaccines.

A micro solid-phase radioimmunoassay (micro-SPRIA) was developed for titration of both anti-HBs and anti-TT antibodies and is described in greater detail below.

A. Humoral Immune Response (Anti-TT) to Tetanus Toxoid

Anti-TT antibodies in mice immunized with TT alone or in association with a hepatitis subunit or polypeptide vaccine were titrated by micro-SPRIA. The geometric mean antibody titers (GMT) of sera obtained from mice immunized with the aluminum-precipitated preparations were significantly higher than the GMT of the sera obtained from animals injected with the corresponding antigens in saline solution for TT ($p < 0.01$; Table 4).

The immunogenicity of TT was decreased in all of the combined antigen preparations. When injected either in saline solution, or coprecipitated on aluminum gel, both P22+TT and HBs+TT vaccines were significantly less immunogenic than TT alone ($p < 0.001$). Anti-TT GMT were similar when comparisons were made between HBs+TT-saline and P22+TT-saline and between HBs+TT-alum and P22+TT-alum.

B. Humoral Immune Response (Anti-HBs) to Hepatitis Polypeptide Vaccine

When injected as a single antigen, P22 precipitated on aluminum gel induced significantly higher anti-HBs titers than P22 in saline solution ($p < 0.001$; Table 5).

The immunogenicity of P22 in saline was significantly increased when administered in conjunction with TT ($p < 0.01$; Table 5). P22+TT-alum also gave significantly higher titers than P22-saline. In contrast, the P22-alum GMT was significantly greater than the GMT observed in animals inoculated with P22+TT in saline or alum ($p < 0.01$).

C. Humoral Immune Response (Anti-HBs) to Hepatitis Subunit Vaccine

The GMT for the aluminum-precipitated HBsAg particles was higher than the GMT for HBsAg particles in saline (Table 6), and both these preparations apparently were more immunogenic than the corresponding polypeptide vaccines (Tables 5 and 6).

When HBsAg particles and TT were administered together in saline solution, the immunogenicity of HBsAg was markedly decreased when compared with the GMT for HBs-saline ($p < 0.02$). However, when both antigens were coprecipitated on alum gel, the GMT was significantly increased when compared to the saline preparation ($p < 0.01$).

These preliminary studies can be summarized as follows. In the case of the HBsAg particle vaccine, when HBsAg and TT were administered as a mixture in saline solution, a mutual competition inhibited the response to both antigens, since both anti-HBs and anti-TT titers were depressed compared to the titers induced by the same vaccines administered alone. When the vaccines were precipitated on aluminum gel, the anti-HBs titers obtained with the antigen mixture were higher than the titers obtained with the individual subunit vaccine, but the opposite was true for anti-TT titers. Under these specific experimental conditions, HBsAg acted as the dominant antigen and TT as the suppressed one.

P22 was the dominant antigen when it was injected together with TT in saline solution, as the anti-HBs titers were significantly higher than those induced by the aqueous polypeptide vaccine alone, while the anti-TT titers were decreased. When P22 and TT were coprecipitated on alum gel, the response to TT was again significantly depressed, as compared to that obtained with TT-alum. Since the anti-HBs response to P22 also was significantly decreased when P22 was associated with alum, both components may be playing a role in suppressing immunogenicity.

III. Development of Anti-HBs Assay with Increased Sensitivity

Since it has been noted previously (11) that HBsAg has the same level of potency in terms of immunogenicity in mouse and man, the studies with the candidate vaccine during the past year have been carried out in mice. We decided to develop a micro solid-phase radioimmunoassay (micro-SPRIA) because routine tests with the commercial Ausab kits were too expensive and required the use of larger volumes of serum.

The final protocol of the micro-SPRIA for mouse anti-HBs is as follows. Purified HBsAg/*adw* was diluted in 0.3 M carbonate-bicarbonate buffer, pH 9.5. Flat-bottom, 96-well plates (polystyrene; Cooke Microtiter System; Dynatech Laboratories, Inc., Alexandria, Va.) were coated with 200 µg of HBsAg/50 µl/well using an overnight incubation at

4°C. The plates were post-coated with 0.5% gelatin (Difco Laboratories, Detroit, Mich.) in 0.05 M PBS, pH 7.2 (gelatin-PBS) for 2 hr at room temperature. Fivefold dilutions of mouse antisera were added in duplicate wells and incubated overnight at 4°C. The plates then were washed 3 times with 0.01% gelatin-PBS. Iodinated goat IgG anti-mouse IgG (^{125}I-GtαM), diluted to 50,000 cpm/50 µl, was added to each well and the plates were further incubated for 2 hr at 37°C. The plates were then washed 3 times as above, sealed, cut off, and counted.

The mouse anti-TT antibodies were titrated in a similar micro-SPRIA. A buffer containing 0.05 M PBS, pH 7.2 + 0.5% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, Mo.) + 0.5% Tween 20 (Sigma) was used instead of gelatin-PBS for washings and for dilution of the ^{125}I-GtαM to decrease background counts. Incubation of the first antibody for 4 hr at 37°C was as efficient as the overnight incubation and was preferred to shorten the test duration.

For both micro-SPRIA, a serum dilution was considered positive if the P/N (counts per minute of the sample/mean counts per minute of 5 negative control mouse sera) was equal to or greater than an arbitrarily chosen cut-off value of 2.1. The titers were calculated by extrapolation of the titration curves, as the reciprocal of the dilution which corresponded to a P/N of 2.1.

We also have developed an enzyme-linked immunoadsorbent assay (ELISA) which utilizes the same goat anti-mouse IgG, coupled to alkaline phosphatase (AP), which was labeled with ^{125}I above. Early comparative tests of these two assays (micro-SPRIA and ELISA) showed that the ELISA was at least 10 times less sensitive than the micro-SPRIA. Our initial AP conjugated antibody reagents had been prepared with a glutaraldehyde cross-linking reaction described earlier (12). In light of the difficulties we have encountered with the uncontrollable activity of glutaraldehyde, we investigated the use of a thio-disulfide bond exchange reaction after reacting both IgG and AP with N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP; Pharmacia) by a method described previously (13). This method has the distinct advantage of allowing control over the molecular size of the resulting conjugate and therefore the specific activity of the conjugated IgG molecule. We have prepared conjugates in which 1 molecule of IgG is linked to 1 molecule of AP.

At this point we compared the relative sensitivities of the micro-SPRIA and ELISA systems with an Ausab test kit for measurement of anti-HBs activity. 23 mouse antisera were tested in the experiment by all three methods; the results are given in Figure 1 and in Table 7. It is obvious that both of our tests offer a greater sensitivity than that afforded by the Ausab test kit. A second point which should be made is that through the use of the newer AP-IgG conjugate, the ELISA is more sensitive than the micro-SPRIA, which is the opposite of that observed with the original glutaraldehyde-prepared conjugate.

IV. Testing of an Alternate Method of Isolation of Purified Polypeptides from Solubilized HBsAg Preparations

A. Polypeptide Purification from HBsAg Solubilized with Mercaptoethanol, Sodium Dodecyl Sulfate, Urea and Heat (2 Min at 100°C)

The work described in this progress report was done with polypeptides isolated by fractionation in 14.5 x 125 mm cylindrical tubes containing 10% polyacrylamide gels (7). Individual protein bands were localized and the gel segments were cut out. The protein

was then extracted from the homogenized gel with 0.05 M PBS. Approximately 60% of the original HBsAg protein was isolated as a P22-P25 pool by this procedure. The major concern with this procedure is that the final product may contain residual fragments of acrylamide gel.

To circumvent the above problem, we fractionated a 20-mg preparation of solubilized HBsAg on a Buchler electrophoretic apparatus as previously described (14). A 15% separation gel was used. The elution pattern is shown in Figure 2. Each fraction was analyzed for distribution of label (^{125}I cpm), for antigenic activity (monitored by Ausria-II testing) and by analytical slab gel PAGE. The elution of protein was similar to that noted previously (14), and antigenic activity was noted throughout. The low antigenic activity was expected since we have previously shown that antigenicity associated with polypeptide populations is relatively inefficient when assayed with a sandwich technique such as Ausria (4). Characterization of each fraction was carried out by analytical slab PAGE. The P22 and P25 polypeptides were localized in fractions 44-57 (Figure 2, solid bar). This represents a yield of 30% of the total protein mass which is free of all gel pieces. Thus, this method may be used to fractionate larger quantities of polypeptides free of extraneous material.

B. Fractionation of Undenatured P22-P25 Polypeptides by the Method Described by Zuckerman et al. (15)

Another approach to obtain P22-P25 HBsAg-derived polypeptides under nondenaturing conditions was by the procedures described by Simons et al. (16) and Skelly et al. (17), with slight modifications.

HBsAg (3 mg) was disrupted under nondenaturing conditions with a final concentration of 2% Triton X-100 in 0.01 M Tris-HCl, 0.5 M NaCl, pH 7.3, for 36 hr at 37°C. ^{125}I-labeled HBsAg was added as tracer before the detergent treatment. The disrupted HBsAg was applied to a 1 x 30 cm column of lentil lectin-Sepharose 4B (Pharmacia Fine Chemicals), equilibrated with 0.01 M Tris-HCl, 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 2% Triton X-100, pH 7.3. The sample was allowed to react with the gel for 30 min at room temperature and then the column was flushed with the above buffer to remove unbound material. The bound material was eluted with 0.01 M Tris-HCl, 0.5 M NaCl, 2% Triton X-100, 5% α-methyl-D-mannoside, pH 7.3. We collected 1-ml fractions and monitored the proteins by OD_{280} and radioactivity.

The data are presented in Figure 3. A major peak of unbound proteins eluted between fractions 12 and 29, and a minor peak of lentil-lectin-bound material eluted with the mannoside buffer. This material is thought to be a soluble complex of P22-P25 which bound to lentil lectin through the glycosylated moiety of P25.

The fractions of the second peak were pooled, dialyzed extensively against 0.01 M Tris-HCl, pH 7.3, at 4°C, and concentrated through an Amicon YM 10 membrane to approximately 4 ml. This material was layered over a preformed linear 20-50% sucrose gradient and spun in an SW41 Beckman rotor at 36,000 rpm for 24 hr. Fractions (0.6 ml) were collected from the top of the tube. Density and HBsAg activity were determined for each fraction. Three peaks were obtained at OD_{280}. HBsAg activity, as measured by Ausria-II, was present in each peak. The top fractions of the peaks (4, 12 and 20) were examined by electron microscopy for the presence of micelles. Fractions 4 and 12 contained almost no micelles. Fraction 20 was loaded with individual and aggregated micelles (Figure 4). The buoyant density of the micelles in sucrose was 1.19 g/cm^3, as compared with a density of 1.17 g/cm^3 reported for 22-nm HBsAg particles in sucrose. The diameter of the micelles ranged from 50 to 250 nm. 30-35% of protein in micelle

form was recovered from the original HBsAg particles, as estimated by protein determination (Lowry method) and percent of radioactivity in peaks. The micelles retained high HBsAg activity, as demonstrated by high P/N ratios obtained by the Ausria test, in contrast to the low activity shown by the polypeptides (see Section IV.A). To further characterize the retention of specific HBsAg activity, the micelles were iodinated using the chloramine-T method and used as labeled antigen for detection of anti-HBs. Two sets of beads from the Ausab kit were first incubated with normal and anti-HBs sera from different species (guinea pig, rabbit and goat) and then one set was incubated with the ^{125}I-labeled HBsAg from an Ausab kit and the second set with ^{125}I-labeled micelle-HBsAg. The P/N ratios of both radioimmunoassays were similar.

V. Evaluation of Cellular Immunity in Hepatitis B Immunized Animals and Humans

We have recently shown that peripheral blood mononuclear cells (PBMCs) from patients who had recovered from HBV infections showed cellular cytotoxicity toward the PLC/PRF/5 HBsAg-producing hepatoma cell line. The effector cell responsible for this effect had the characteristics of a natural killer cell. Similar studies were instituted in mice and guinea pigs and in 20 young adult volunteers who were vaccinated with purified HBsAg in a study on the reactogenicity and immunogenicity of an HBV vaccine. The HBsAg vaccine used in the human studies consisted of purified, formalin-inactivated, alum-adsorbed HBsAg, subtype *adw*. In some individuals, blood was initially obtained before the first vaccine dose was given. Subsequent samples were then drawn after circulating levels of anti-HBs antibodies were detected. In five subjects, however, no production of antibodies had developed after three inoculations of 40 µg of HBsAg each dose. Blood was also obtained from these individuals and tested for the production of suppressive factors as described below.

Female BALB/c mice were immunized with 40-160 µg of purified HBsAg intraperitoneally either once or twice. One to two months after their last inoculation, an intravenous booster of 5-20 µg HBsAg was given and the mice were sacrificed 3 days later. Before they were sacrificed, blood was obtained and the presence of circulating anti-HBs antibodies was confirmed by radioimmunoassay. These experiments were conducted in conjunction with studies on monoclonal antibody production for HBsAg. A total of 6 immunized mice and 4 control mice (immunized with tetanus toxoid) were tested for lymphocyte cytotoxicity toward the PLC/PRF/5 or Mahlavu cell lines. None showed cytotoxicity toward any of the targets (data not shown). In contrast, two mice that were immunized with 10×10^6 PLC/PRF/5 cells twice intraperitoneally did possess cytotoxicity toward both the PLC/PRF/5 and Mahlavu cell lines (data not shown). Interestingly, these mice did not develop anti-HBs antibodies. Most likely, the target antigens were human hepatocyte antigens common to both cell lines. Further experiments examining this xenogeneic system were not performed since the original purpose was to determine whether lymphocytes from immunized mice could show specific cytotoxicity. Positive results would have led to determination of the cells responsible and hopefully would have complemented the results obtained with humans.

Previous studies performed by Cabral et al. (7, 18) and Sanchez et al. (10) showed the presence of cellular immunity to HBsAg in immunized guinea pigs. Spleens were obtained from guinea pigs that had been inoculated with purified HBsAg or one of its polypeptides (P68). Spleen cells from these animals also did not show cytotoxicity toward the human hepatoma cell lines, although circulating levels of anti-HBs were present (data not shown).

Three human subjects who had been passively immunized with commercial high-titered anti-HBs globulin (H-BIG) were studied. All had been exposed to HBV-contaminated material and were therefore at high risk for subsequent HBV infection. All developed moderately high amounts of anti-HBs antibodies after intramuscular inoculation of H-BIG [mean radioimmunoassay positive-to-negative (P/N) of 58.4 ± 8.6]. Two to four weeks after inoculation of H-BIG, peripheral blood lymphocytes were obtained and studied for cytotoxicity. No specific cytotoxicity was observed (data not shown), although lymphocytes from one subject did show nonspecific cytotoxic activity toward both cell lines which did not persist after an overnight incubation at 37°C.

About 200 healthy young adult volunteers were participating in a separate study on the immunogenicity of a purified, formalin-inactivated HBsAg vaccine. 20 of these individuals were studied for lymphocyte cytotoxicity. Three subjects had been tested before the first dose. No difference in response was observed upon vaccination with HBsAg, although circulating levels of anti-HBs were present. In 15 individuals, no significant cytotoxicity was observed above a background of 5-15% specific lysis. In two, however, cytotoxicity was observed which was specific for the PLC/PRF/5 target cells and appeared to be dose-dependent. Finally, 5 subjects who did not respond with the production of specific antibody after 3 inoculations were also not cytotoxic (data not shown).

VI. Propagation of Hepatitis A Virus (HAV) in Cell Cultures: Proteolytic Enhancement

Our initial plan was to study mechanisms of replication for HAV that might enhance the infectivity of HAV using (i) proteolytic enzymes (pancreatin and trypsin) which are known to exert their activity on virus particles by converting noninfectious particles to infectious virions, and (ii) DEAE-dextran, a polycationic, diethylaminoethyl ether of dextran which stimulates cellular uptake of nucleic acids and enhances plaque formation by viruses and infective RNA.

Continuous cell lines have been propagated and cell stocks stored in liquid nitrogen: (i) MA-104 — received from Dr. M.K. Estes and stored at passage 57; (ii) BGMK — stored at passage 53; (iii) PLC/PRF/5 hepatoma cell line (HBsAg secretor) — courtesy of Dr. Stanley Lemon, Walter Reed Army Institute of Research; (iv) Mahlavu hepatoma cell line (HBsAg nonsecretor) — courtesy of Dr. I. Millman; (v) MRC-5, (vi) HEF and (vii) Hep-2 — all purchased from Microbiological Associates.

BGMK cells have been cultivated in MEM containing 20 mM HEPES, 0.225% $NaHCO_3$, 0.25% lactalbumin hydrolysate, 0.5% bovine serum albumin, antibiotics, and glutamine in the absence of serum for a period of at least 1 month without adverse effects when media was changed weekly.

Marmoset-adapted HAV was prepared from about 5 grams of marmoset liver by either of two methods.

(1) Procedure I (Figure 5): Initial studies have shown that when HAV-infected liver was homogenized in glycine-HCl, pH 1.5, and then layered over 30% sucrose prepared in an identical buffer system prior to centrifugation at 24,000 rpm for 18 hr at 4°C, antigen/antibody complexes presumably can be dissociated and/or kept from forming. Figure 6 shows the results obtained using this procedure.

(2) Procedure II (Figure 7): Marmoset liver tissue was homogenized with PBS, pH 7.4, containing 0.05 M phosphate, saline, EDTA and Triton X-100. The homogenate was sonicated for 30 sec and centrifuged at 5000 rpm for 15 min. The supernatant fluid was collected, and the pellet was resuspended in 20 ml of PBS buffer, sonicated and recentrifuged. The supernatant fluids were then combined. Fractions were layered over 30% sucrose and a cushion of 60% sucrose. Centrifugation was performed at 25,000 rpm for 15 hr at 4°C in an SW27 rotor. Fractions were collected from the bottom of the centrifuge tube and were tested by radioimmunoassay for HAV antigen. Figure 8 shows the results obtained with this procedure.

In addition, HAV has been isolated from fecal extracts of chimpanzees infected with HAV. Two procedures have been used.

(1) Procedure III: 2.0 ml of a 20% chimpanzee fecal suspension containing HAV was mixed with 1.0 ml lactalbumin hydrolysate and filtered through a 45-μm filter. All samples were stored at -30°C until used.

(2) Procedure IV: A 20% fecal suspension was clarified, and HAV was isolated using 10% PEG 6000. The pellet was resolubilized with PBS and the sample was placed on a CsCl gradient and subjected to isopycnic centrifugation. Fractions were collected and those containing the bulk of the HAV antigen were pooled for use as an inoculum.

BGMK cells (passage 63) were treated with DEAE-dextran (10 or 100 μg/ml) for 30 min at 37°C. Partially purified marmoset-adapted HAV and HAV from fecal extracts (Procedure III) were incubated for 60 min with a solution of trypsin (10 μg/ml) prepared in Hank BSS. The virus-trypsin samples were diluted 10-fold (10^{-1}, 10^{-2} and 10^{-3}) to diminish the toxic effect of the trypsin, then added to the DEAE-dextran-treated BGMK cells. After an incubation period of 60 min at 37°C, the plates were washed and maintenance media, without trypsin, was added to the cells. Control cells received trypsin solution only. Coverslips are being evaluated weekly for HAV by immunofluorescence.

In another set of experiments, five cell lines (PLC/PRF/5, Mahlavu, MRC-5, HEF and Hep-2) were infected with HAV obtained by Procedure IV. This material was inoculated initially into a primary cell culture derived from a neonatal hepatic carcinoma. After 1 week, the cells were scraped from the plate, sonicated, and the clarified supernatant fluid used as inoculum for the previously described cell lines. Table 8 shows the results of these studies. During week 5, evidence of HAV replication or expression was detected in the PLC/PRF/5 and MRC-5 cell lines when compared with the uninfected cells.

VII. Detection of HAV and Rotavirus in a Community Water Supply Following an Outbreak of Gastroenteritis and Infectious Hepatitis

Georgetown, Texas (population 13,000), is one of many small communities in the United States which uses a groundwater supply with no water treatment except chlorination. Therefore, if the raw water supply is unexpectedly contaminated, the community is susceptible to a waterborne disease outbreak. In a review of the problem of viruses in groundwater, approximately 50% of the documented outbreaks of waterborne disease in the United States were noted to be related to contaminated groundwater (19).

In June 1980 an outbreak of gastrointestinal illness characterized by an acute onset of diarrhea, abdominal cramps, nausea and fever occurred in Georgetown, Texas. Illness was associated with drinking water from the central city wells in Georgetown. The attack

rate among the 10,000 individuals living in areas supplied by these wells was approximately 79%. An increased number of cases of hepatitis A began to appear in July 1980. 29 cases of hepatitis were reported from July 6-15, with a total of 36 cases reported during the month. This compared to an expected incidence of 0-2 cases of hepatitis A per month for the Georgetown area.

Georgetown receives its water from 7 wells. There are 4 central city wells ranging from 186 to 210 feet deep which pump water to a common 750,000-gallon storage reservoir. Georgetown is situated in a limestone region and draws its water from the Edwards aquifer. Due to the porous nature of the aquifer and the fact that the aquifer is exposed to the surface by a fault line running just west of Georgetown, several possibilities exist for contamination of the wells. These include: (i) leakage from nearby sewer lines; (ii) contamination from nearby abandoned or private wells or septic tanks; and (iii) relatively long-distance contamination from surface contaminants in the recharge area of the Edwards aquifer.

During the second peak of gastroenteritis cases, samples of sewage, well water and tap water were taken for virological analysis. Well and tap water samples of 400-1,000 liters were concentrated by filtration through either positively charged depth filters (1-MDS, AMF/CUNO, Meriden, Conn.) or negatively charged filters (Duo-Fine, Filterite Corp., Timonium, Md.). With the Filterite filters it was necessary to adjust the pH to 3.5 by injecting 1 N HCl into the sample before filtration. Aluminum chloride was also added through the injection port to a final concentration of 0.005 M. These conditions allow for maximum adsorption of the virus to the Filterite filters (20). With the 1-MDS filters no additives were necessary since the positively charged filters adsorb the negatively charged virus particles at ambient conditions. When tap water samples were taken, a sodium thiosulfate solution was injected ahead of the filter to neutralize the residual chlorine.

Each filter cartridge was eluted with 1 liter of 3% beef extract at pH 10.0. This eluate was concentrated by an organic flocculation technique (21). Briefly, each eluate was adjusted to pH 3.5 using 1 M glycine, pH 1.5. The floc that formed was collected by centrifugation, and the virus that adsorbed to this floc was eluted with 0.05 M glycine at pH 9.5. The resulting suspension was then clarified by centrifugation, treated with antibiotics, and inoculated onto BGM cell monolayers to assay for enterovirus (22). An aliquot of each concentrate was further concentrated by ultracentrifugation to assay for HAV antigen and rotavirus. Samples were assayed for rotavirus using an indirect immunofluorescence test (23) and for HAV antigen using a radioimmunoassay (24, 25).

Table 9 shows the concentrations of enterovirus in sewage, well water and tap water samples taken on June 19. The concentration of enterovirus in the five sewage samples ranged from 1,200 to 7,400 plaque-forming units (PFU) per 100 liters, giving an average of 4,380 PFU/100 liters.

Rotavirus, a major cause of infantile diarrhea, was detected in two sewage samples at a low level. Rotavirus was not detected in any of the well or tap water samples that were tested. Seven fecal specimens from acutely ill Georgetown residents were tested and were negative for rotavirus.

Table 10 shows the viral isolates that were obtained from each sample location. From sewage samples, 20 of 42 plaques were confirmed; 19 were identified as coxsackievirus B3 (CB3) and 1 as coxsackievirus B2 (CB2). A total of 26 virus-like plaques were harvested from cells inoculated with samples from Georgetown's central wells (no. 3 and 4); 7 of these were identified, with 6 identified as CB2 and 1 as CB3. The two isolates

from the tap water were both identified as CB3. When the well and tap water were tested again on July 27, no viruses were isolated. Serologic studies indicate that CB3 could have been partly responsible for the Georgetown outbreak.

HAV antigen was detected in 3 of 5 of the sewage concentrates and in 1 well water concentrate from samples taken on June 19. This preceded the outbreak of hepatitis cases, which occurred in mid-July. Table 11 summarizes the hepatitis results. The positive sewage and well water samples were neutralized by human IgG from a convalescent hepatitis patient. The convalescent IgG did not react with CB2 or CB3 isolated from the same samples or with other common enteroviruses. The buoyant density of the sewage isolates in a CsCl gradient was 1.34 g/ml, which is in the range reported for HAV particles.

Human enteric viruses were isolated from the potable water despite a total chlorine residual of 0.8 mg/liter. This is significant since the bacteriological samples of tap water taken by local and state officials were consistently free of coliform bacteria. The bacteriological results led city officials to believe that the water was safe when, in fact, viruses were surviving the chlorination process.

There are at least two other reports of gastroenteritis outbreaks related to potable water which met bacteriological standards. Wellings et al. (26) isolated echovirus 22/23 from a disinfected groundwater supply during an outbreak at a migrant labor camp in Florida. More recently, an iodinated (0.7-1.0 ppm) groundwater supply was implicated in an outbreak of viral gastroenteritis at a summer camp in Maryland (27), even though drinking water samples taken before, during and after the epidemic met bacteriological standards. These findings support the recommendations recently made by a Scientific Committee of the World Health Organization (28) and by an American Water Works Association T&P committee (29) which include standards for viruses in water and possible monitoring of viral contamination in certain situations.

The Georgetown incident reemphasizes that the purity of groundwater cannot always be relied on. Barriers against contamination of potable water supplies in communities that depend on groundwater must be properly maintained and adequate for intermittent contamination. These barriers may need to be stronger in certain areas, such as limestone regions, which may be especially susceptible to contamination from distant sources.

The ability to detect hepatitis A virus antigen in a water source prior to an outbreak could be valuable in the prevention of future outbreaks. Prophylactic treatment of the population with gammaglobulin upon discovery of hepatitis in a water source could decrease the severity of the outbreak. Unfortunately, in the Georgetown epidemic, the samples were not assayed for hepatitis until after the outbreak, so no preventive measures were taken. In the future, rapid analysis of water samples for hepatitis A virus may aid in reducing the number of cases of morbidity and mortality from waterborne hepatitis.

VIII. Preliminary Testing of Polypeptide Vaccine and Synthetic Peptide Vaccine in Mice

Preliminary results (Table 12) are available which strongly support the likelihood of success for our proposed work scope for the new contract year. Groups of mice were immunized with 1 dose of two difference types of vaccine. The first type was the micelle preparation prepared according to the Zuckerman procedure. The second type of vaccine involves injection of the first two sets of synthetic peptides made by Dr. Sparrow (Baylor College of Medicine). These two peptides consist of amino acid residues 117-137 (20 residues) and amino acid residues 122-137 (15 residues).

It is evident that the micelle polypeptide preparation is highly immunogenic (Table 12). In keeping with their report (Zuckerman et al., in Hepatitis B Vaccine, pp. 251-262, Elsevier-North Holland Biomed. Press) the same dose of micelles produced higher levels of antibody than observed in mice immunized with intact particles (Tables 1 and 12). It was of interest that the antibody levels dropped between days 7 and 14 after inoculation of micelles suspended in saline, whereas the titers increased during the same time period in those mice inoculated with micelles precipitated in alum (Table 12).

The results of our preliminary work with synthetic peptides (117-137 and 122-137) are most exciting. In every group of mice injected, regardless of the adjuvant vehicle employed, at least one-half of the mice in each group produced detectable levels of antibody at a serum dilution of 1:4, after 1 dose of peptide. In fact, in almost all cases the antibody response was equal to that seen in mice injected with SDS-denatured P22 polypeptide material (Tables 2 and 12).

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TABLE 1

Anti-HBs Activity in Mice Immunized with NIAID Subunit Vaccine

Mouse no.	P/N values (Ausab)[a]				
	Days after inoculation:				
	13	31	73[b]	94 (17)[c]	111 (34)
	Alum-precipitated subunit vaccine				
1	45	91	113	310	254
2	34	63	70	305	252
3	28	31	60	268	240
4	24	29	57	245	232
5	22	27	56	173	174
6	18	24	29	162	173
	Aqueous subunit vaccine				
1	13	11	31	265	301
2	11	9	11	202	149
3	7.7	5.4	10	157	140
4	3.7	2.3	8.4	89	86
5	2.2	1.9	1.7	25	22

[a] All sera were tested at a 1:4 dilution.

[b] On day 73, all mice were bled and subsequently inoculated with a booster dose.

[c] Numbers in parentheses indicate days following booster inoculation.

TABLE 2

Anti-HBs Activity in Mice Immunized with a P22-P25 Polypeptide Pool Derived from the NIAID Vaccine

Mouse no.	P/N values (Ausab)[a] Days after inoculation: 7	14	21[b]	33 (12)[c]	51 (30)	133 (112)
	Alum-precipitated, formalin-treated polypeptide vaccine					
1	1.5	1.6	0.8	1.3	0.8	-[d]
2	1.3	0.8	0.6	0.6	0.5	-
3	1.1	2.0	1.2	0.8	1.2	-
4	1.0	1.4	0.7	1.6	0.7	-
5	2.9	2.5	1.2	1.5	1.2	-
6	1.5	0.9	1.4	-	-	-
	Alum-precipitated, untreated polypeptide vaccine					
1	3.1	2.5	0.8	1.3	1.4	1.4
2	3.0	2.9	0.7	1.3	0.8	1.2
3	4.6	3.2	1.9	1.4	0.8	2.6
4	2.1	1.3	0.8	0.7	0.7	1.2
5	8.6	7.4	9.1	5.6	4.2	5.6
6	6.1	4.8	4.3	3.3	2.1	3.0

[a] All sera were tested at a 1:4 dilution.

[b] On day 21, all mice were bled and subsequently inoculated with a booster dose. Both groups of mice were boostered with formalin-treated polypeptide vaccine.

[c] Numbers in parentheses indicate days following booster inoculation.

[d] Mice not available at that time.

TABLE 3

Anti-HBs Activity in Mice Immunized with a P22-P25 Alum-Precipitated Polypeptide Vaccine after Storage for 2 Months

Mouse no.	P/N values (Ausab)[a]		
	Days after inoculation		
	10	21	72
1	1.7	2.7	3.0
2	2.8	2.4	2.1
3	1.0	0.9	2.9
4	1.6	3.0	3.8
5	4.3	2.4	3.0
6	3.3	2.5	1.4
7	5.4	0.6	[b]

[a] All sera were tested at a 1:4 dilution.

[b] Mouse not available at that time.

TABLE 4

Anti-Tetanus Toxoid Titers in Mice Immunized with Tetanus Toxoid Alone or in Association with a Hepatitis Subunit or Polypeptide Vaccine

Preparation	No. mice tested	Titer range[a]	Geometric mean titer
TT-saline	4	160,000 - 640,000	378,296
TT-alum	5	30,000,000 - 60,000,000	42,875,516
P22+TT-saline	6	94,000 - 640,000	269,827
P22+TT-alum	6	1,900,000 - 3,800,000	2,822,768
HBs+TT-saline	6	31,250 - 700,000	196,217
HBS+TT-alum	6	781,250 - 7,600,000	1,892,364

[a] Titers are expressed as the reciprocal of that serum dilution which would correspond to a P/N ratio of 2.1 in micro-SPRIA.

TABLE 5

Anti-HBs Titers in Mice Immunized with Polypeptide Vaccine in Association with Tetanus Toxoid

Preparation	No. mice tested	Titer range[a]	Geometric mean titer
P22-saline	6	1,500 - 18,000	5,647
P22+TT-saline	6	15,000 - 115,000	41,917
P22-alum	6	52,000 - 580,000	236,678
P22+TT-alum	6	22,000 - 220,000	48,154

[a] Titers are expressed as the reciprocal of that serum dilution which would correspond to a P/N of 2.1 in micro-SPRIA.

TABLE 6

Anti-HBs Titers in Mice Immunized with Subunit HBsAg Vaccine in Association with Tetanus Toxoid

Preparation	No. mice tested	Titer range[a]	Geometric mean titer
HBs-saline	6	230,000 - 720,000	457,748
HBs+TT-saline	5	18,000 - 350,000	67,272
HBs-alum	4	440,000 - 1,050,000	794,857
HBs+TT-alum	6	540,000 - 3,000,000	1,076,794

[a] Titers are expressed as the reciprocal of that serum dilution which would correspond to a P/N ratio of 2.1 in micro-SPRIA.

TABLE 7

Comparative Sensitivities of Antibody Assays for Anti-HBs Activity

Assay	Mean titer[a]	Sensitivity
Ausab[b]	250	1
Micro-SPRIA	51,000	204
ELISA	89,268	357

[a] Geometric mean titer of 23 individual mouse anti-HBs sera.

[b] Commercial RIA kit purchased from Abbott Laboratories.

TABLE 8

Solid-Phase (Microtiter) RIA for HAV Antigen
Detection in Supernatants from Five Human Cell Lines

Cell line		S/N ratio in supernatant		
		Weeks after infection:		
		3	4	5
PLC/PRF/5	infected	1.03	2.24	3.24
	not infected	1.15	1.20	1.27
Mahlavu	infected	1.30	1.87	2.87
	not infected	1.29	1.08	1.59
MRC-5	infected	1.16	1.09	3.07
	not infected	1.11	1.16	1.00
HEF	infected	1.10	1.44	1.69
	not infected	1.10	ND[a]	ND
Hep-2	infected	ND	1.88	1.90
	not infected	1.39	1.60	ND
Chimp stool (control S/N values for each set of assays)		6.19	7.80	14.20

[a] ND = Not done.

TABLE 9

Concentrations of Enterovirus in Georgetown Sewage, Well Water and Tap Water on June 19, 1980

Sample	Type	Filter	Volume (gal)	Enterovirus	
				No. PFU observed[a]	PFU/ 100 liters
G1	sewage	Filterite	25	14	6,400
G2	sewage	Filterite	25	7	4,300
G3	sewage	Filterite	25	12	7,400
G4	sewage	Filterite	25	6	3,600
G5	sewage	1-MDS	27	3	1,200
G6	well no. 4	1-MDS	250	21	13
G7	well no. 4	Filterite	100	0	-
G8	well no. 3	1-MDS	250	4	3
G9	tap (WTP)[b]	1-MDS	190	2	1
G10	tap (well no. 5)	1-MDS	240	0	-
G11	tap (OTP)[c]	Filterite	200	0	-

[a] Total plaque-forming units (PFU) observed; only a portion of each concentrate was inoculated.

[b] WTP = Water treatment plant.

[c] OTP = Old town park.

TABLE 10

Virus Isolates from Sewage, Wells and Tap Water in Georgetown

Sample	Type	Date	Volume (gal)	No. plaques harvested	No. confirmed	Viruses identified[a]
G1-G5	sewage	6/19/80	-	42	20	CB3 (19); CB2 (1); HAV; rotavirus
G6	well no. 4	6/19/80	250	21	5	CB3 (1); CB2 (4)
G8	well no. 3	6/19/80	250	5	2	CB2 (2); HAV
G9	tap (WTP)[b]	6/19/80	190	2	2	CB3 (2)
G10	tap (well no. 5)	6/19/80	240	0	0	-
G11	tap (OTP)[c]	6/19/80	200	0	0	-
G12	well no. 4	7/27/80	300	0	0	-
G13	tap (WTP)	7/27/80	477	0	0	-
G14	well no. 3	7/27/80	843	0	0	-
G15	tap (well no. 5)	7/27/80	330	0	0	-

[a] HAV and rotavirus were identified by radioimmunoassay or immunofluorescent staining, respectively. The number of isolates is given in parentheses.

[b] WTP = Water treatment plant.

[c] OTP = Old town park.

TABLE 11

Detection by Radioimmunoassay of HAV Antigen
in Georgetown Sewage and Well Water

Sample	Type	Enterovirus	HAV antigen	
			Sample only P/N[a]	Neutralized P/N[b]
G1	sewage	+	1.88	1.59
G2	sewage	+	3.45 (+)	1.67
G3	sewage	+	1.81	1.77
G4	sewage	+	3.91 (+)	1.19
G5	sewage	+	4.36 (+)	1.99
G6	well no. 4	+	0.90	1.00
G8	well no. 3	+	2.30 (+)	1.19
G9	tap	+	1.06	ND[c]
G10	tap	-	0.93	1.06
G11	tap	-	1.78	ND

[a] Ratio of sample to negative control. A ratio >2.0 indicates a positive test (+).

[b] Ratio of sample after reaction with human IgG anti-HAV to negative control. The sample was reacted with a specific antibody prior to testing by radioimmunoassay.

[c] ND = Not done.

TABLE 12

Anti-HBs Activity in Mice Immunized with a Micelle Polypeptide Preparation (Groups A and B) and with a Synthetic HBsAg Peptide (Groups C-H)

Group	Preparation	Antibody activity[a] at day 7		Antibody activity[a] at day 14	
		Positive/total	P/N range	Positive/total	P/N range
A	micelle - saline	5/5	19-99	5/5	8-43
B	micelle - alum	6/6	19-131	6/6	53-220
C	117-137 - FCA	4/6	3.1-6.2	5/6	2.9-7.3
D	122-137 - FCA	3/6	3.2-4.5	3/6	4.6-8.2
E	117-137 - alum	2/6	2.8-5.4	4/6	2.1-8.6
F	122-137 - alum	4/6	2.3-9.8	4/6	2.5-7.0
G	122-137 - liposomes[b]	3/6	3.2-6.4	3/6	3.5-8.3
H	122-137 - liposomes[b] + MDP	4/6	2.3-15.2	3/6	2.8-13.6

[a] Positive antibody activity at P/N value of $\geq$2.1 at serum dilution of 1:4 when tested by Ausab.

[b] Liposome - synthetic peptides entrapped in liposomes.

FIGURE 1. Comparative sensitivities of testing of 23 individual mouse sera by different assays for anti-HBs.

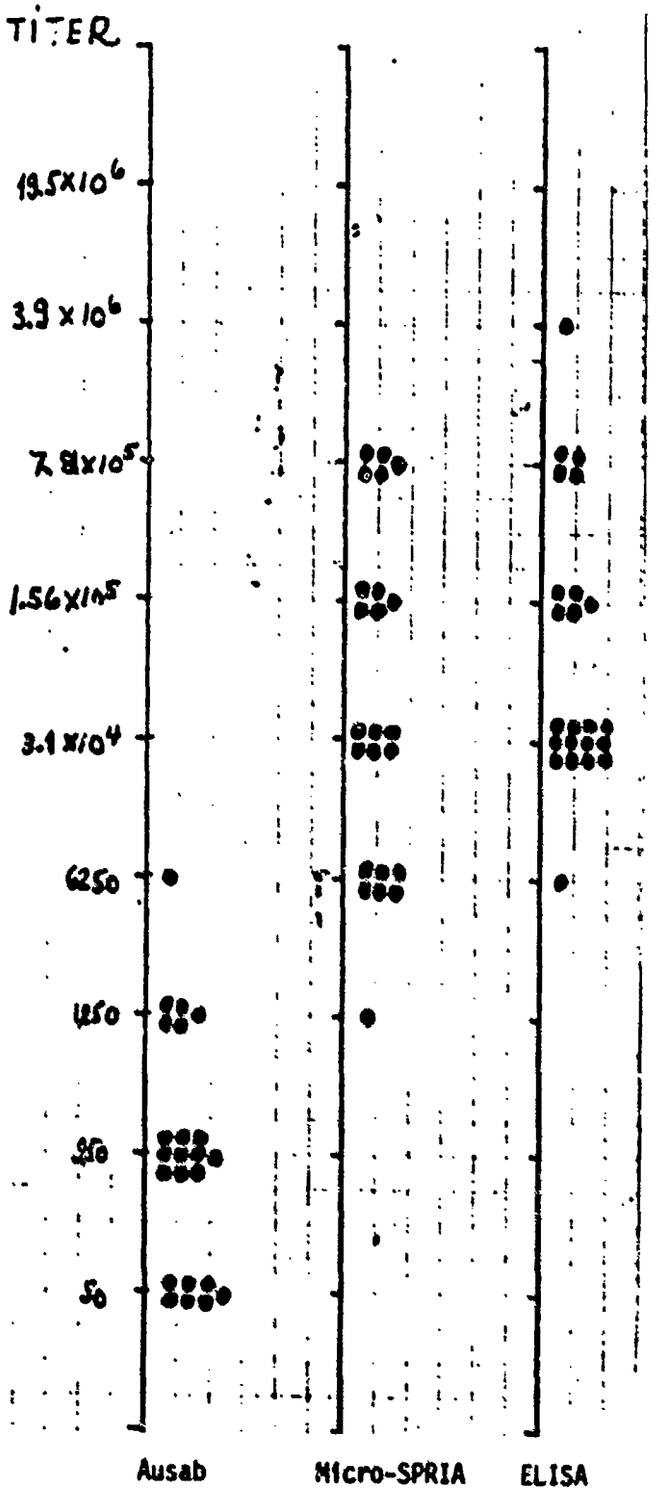


FIGURE 2. Elution profile of 20 mg of HBsAg/adw trace labeled with ^{125}I, solubilized in SDS, urea, and mercaptoethanol by heating at 100°C for 2 min, from preparative PAGE utilizing 15% polyacrylamide gels. A pool of fractions was made, as indicated by the heavy bar, which contained the P22 and P25 polypeptides.

Figure 2.

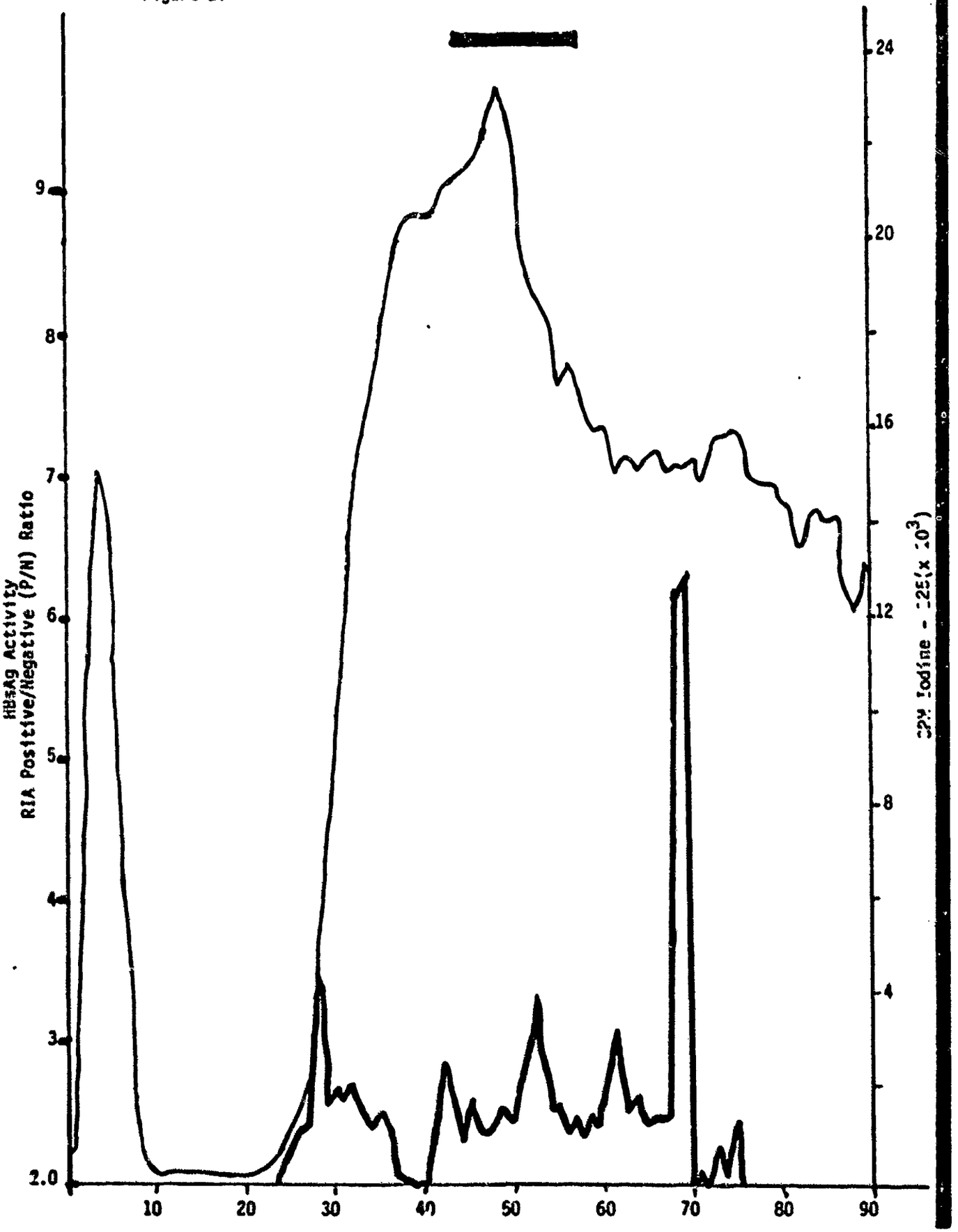

HBsAg Activity
RIA Positive/Negative (P/N) Ratio
9
8
7
6
5
4
3
2.0
24
20
16
12
8
4
10
20
30
40
50
60
70
80
90

FIGURE 3. Affinity chromatography of 5 mg disrupted HBsAg on lentil lectin-Sepharose 4B. The antigen was disrupted and the unbound material was removed from the column by washing with the starting buffer (0.01 M Tris-HCl, pH 7.3, containing 2% Triton X-100, 0.5 M NaCl, 1 mM $MnCl_2$, and 1 mM $CaCl_2$). The arrow indicates the start of elution of the bound material with 0.01 M Tris-HCl, pH 7.3, containing 2% Triton X-100, 0.5 M NaCl, and 5% α-methyl-D-mannoside.

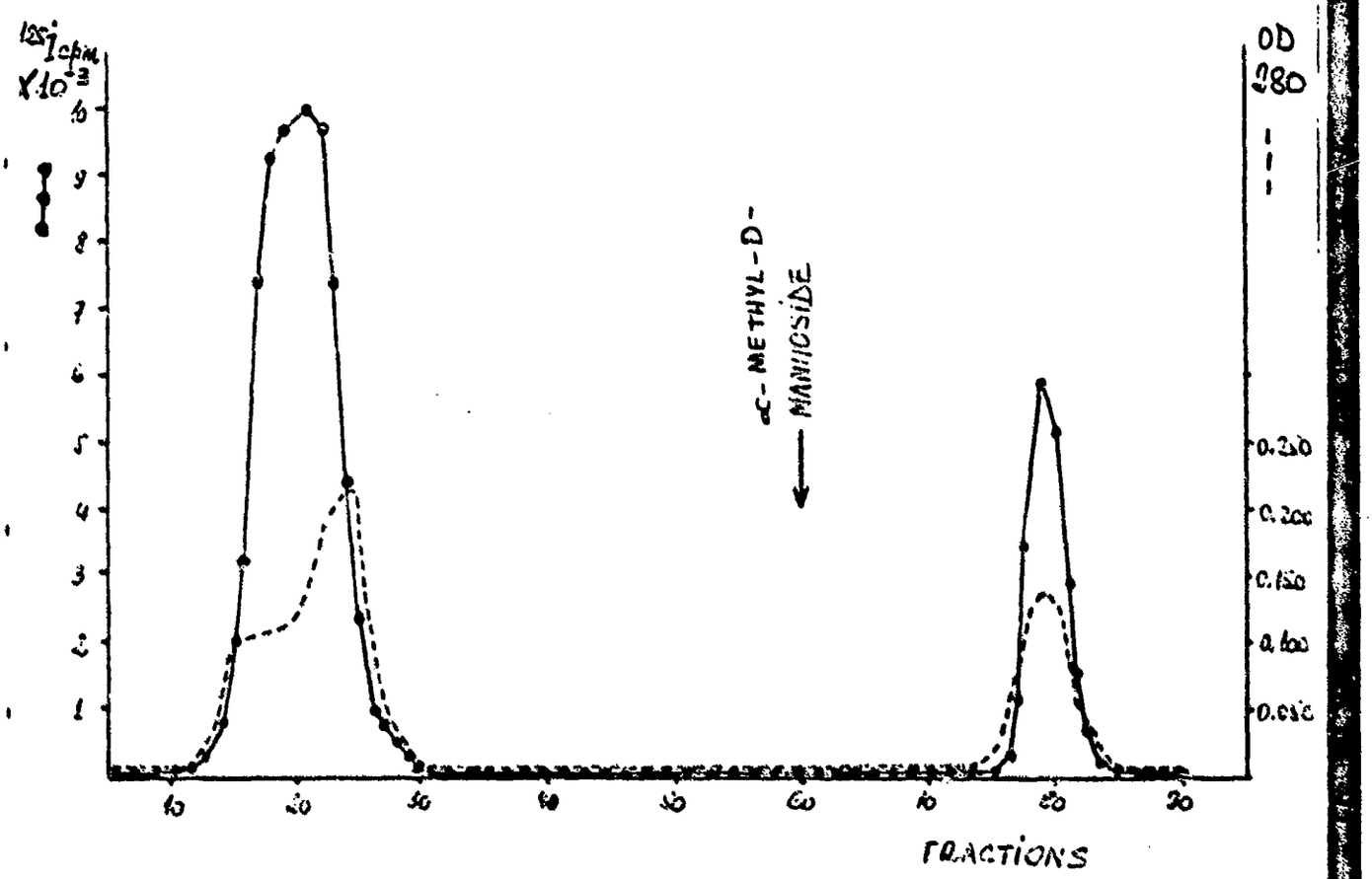


FIGURE 4. Electron micrographs of micelles of HBsAg. Density 1.19 g/cm^3 in sucrose. Bar represents 200 nm.

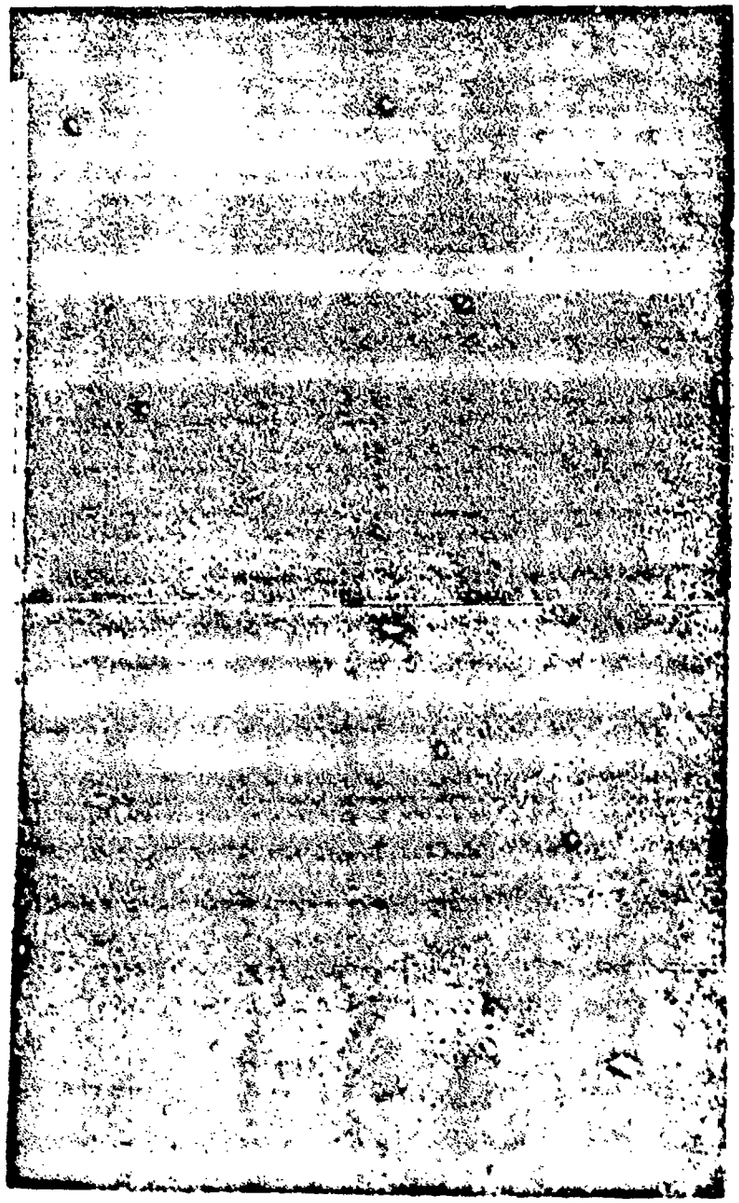


FIGURE 5. Procedure I: Schematic presentation for isolation and purification of HAV from chimpanzee liver at pH 1.5

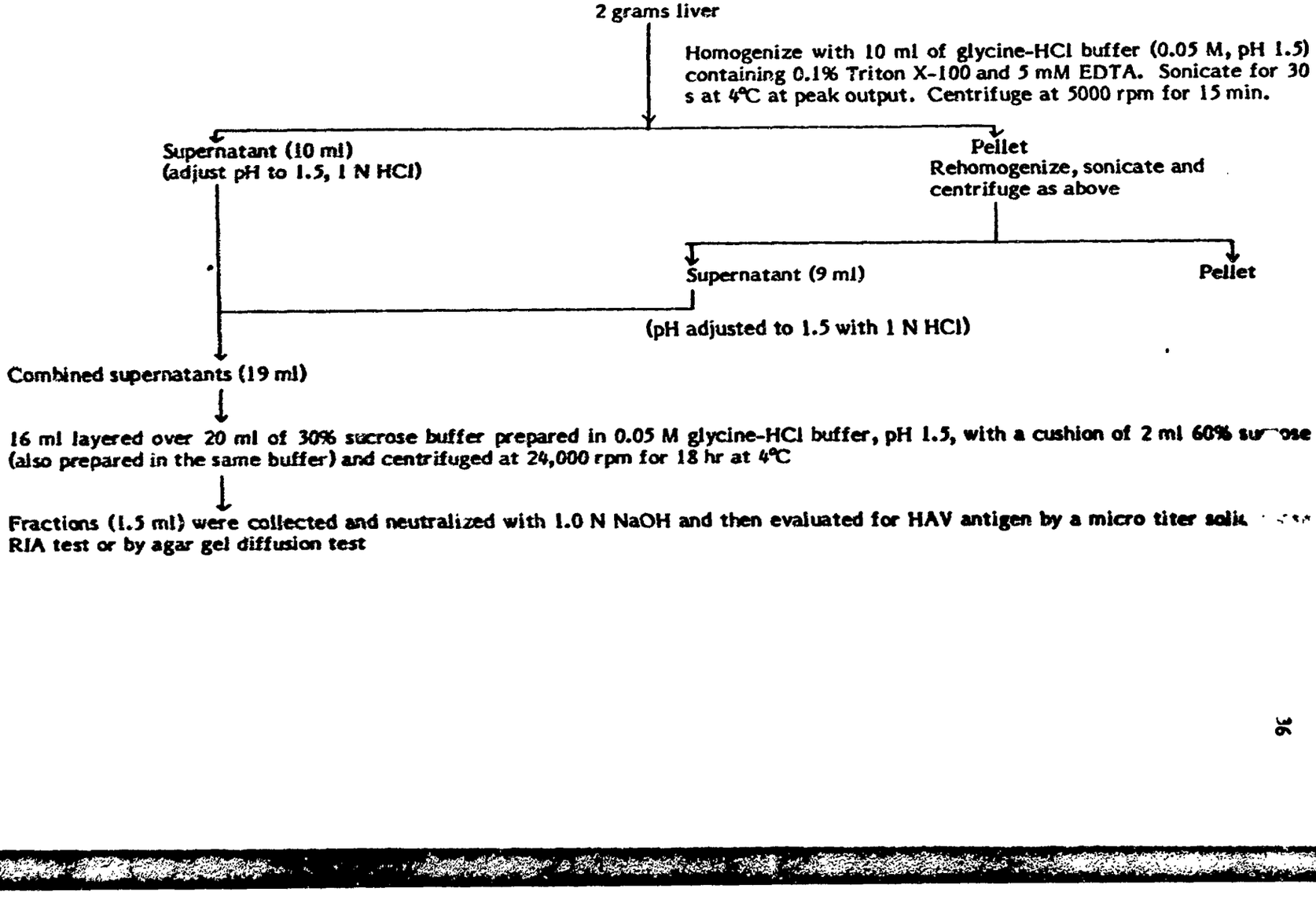


FIGURE 6. Extraction of HAV at low pH from infected marmoset liver

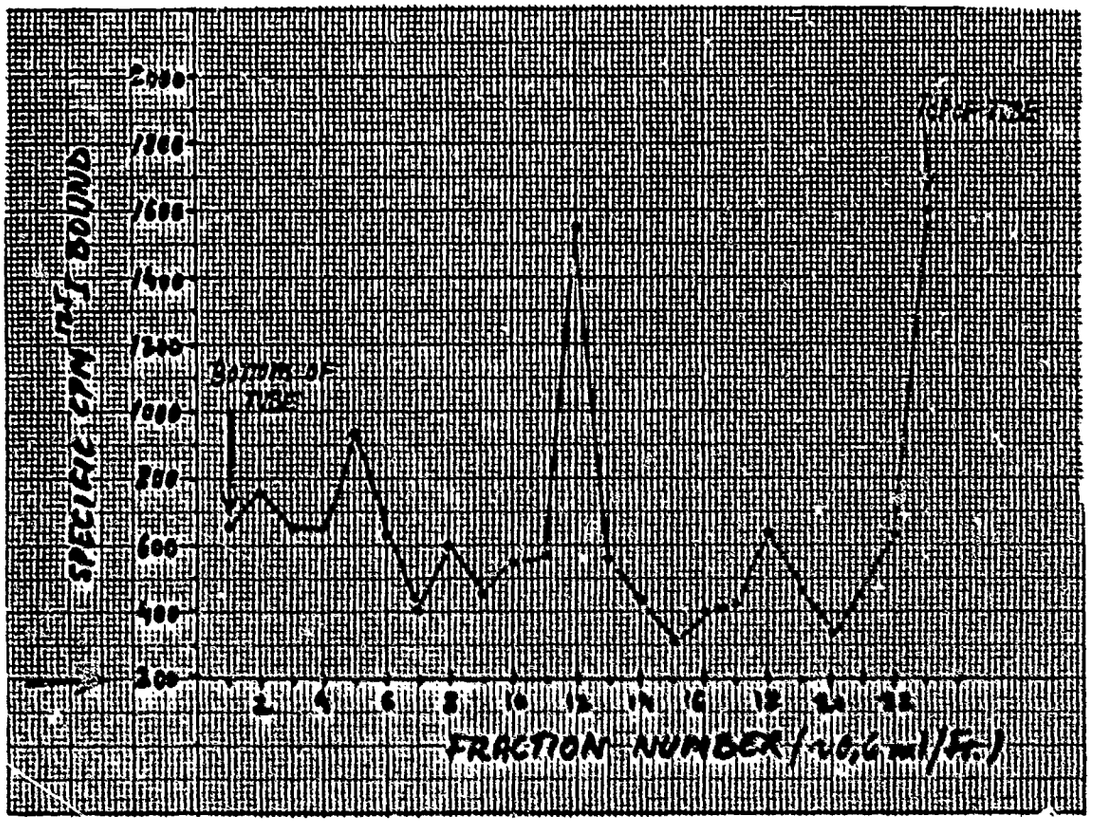


FIGURE 7. Procedure II: Schematic presentation for isolation and purification of HAV antigen from chimpanzee liver

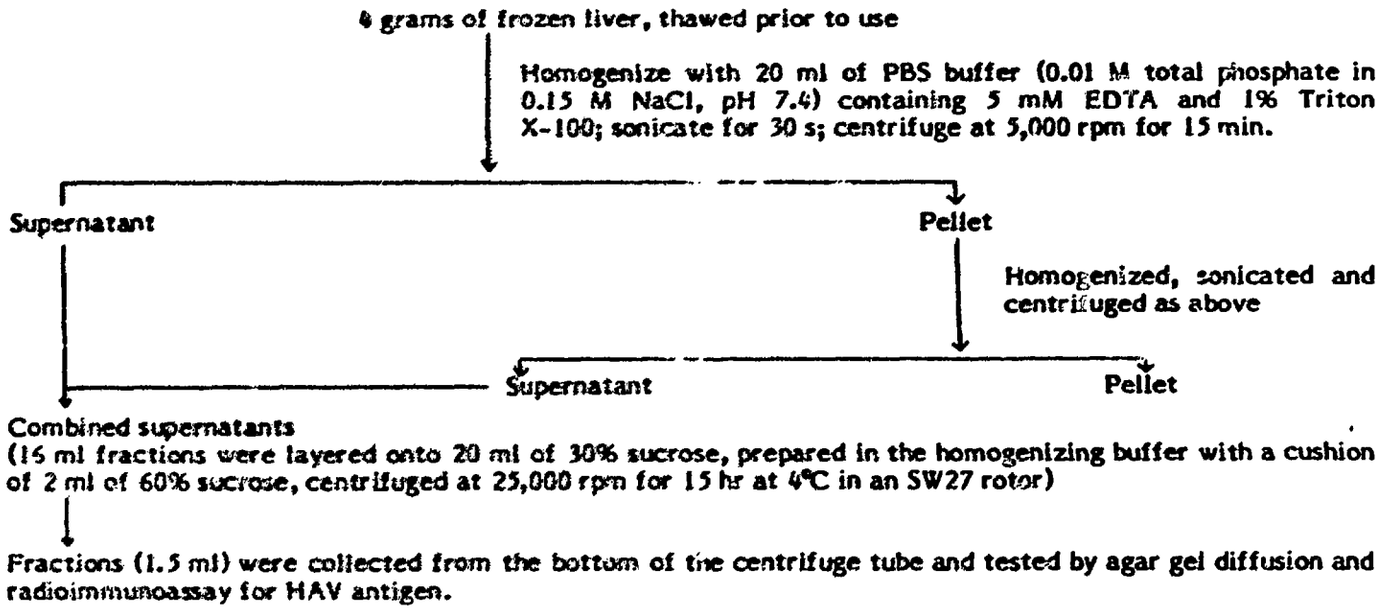
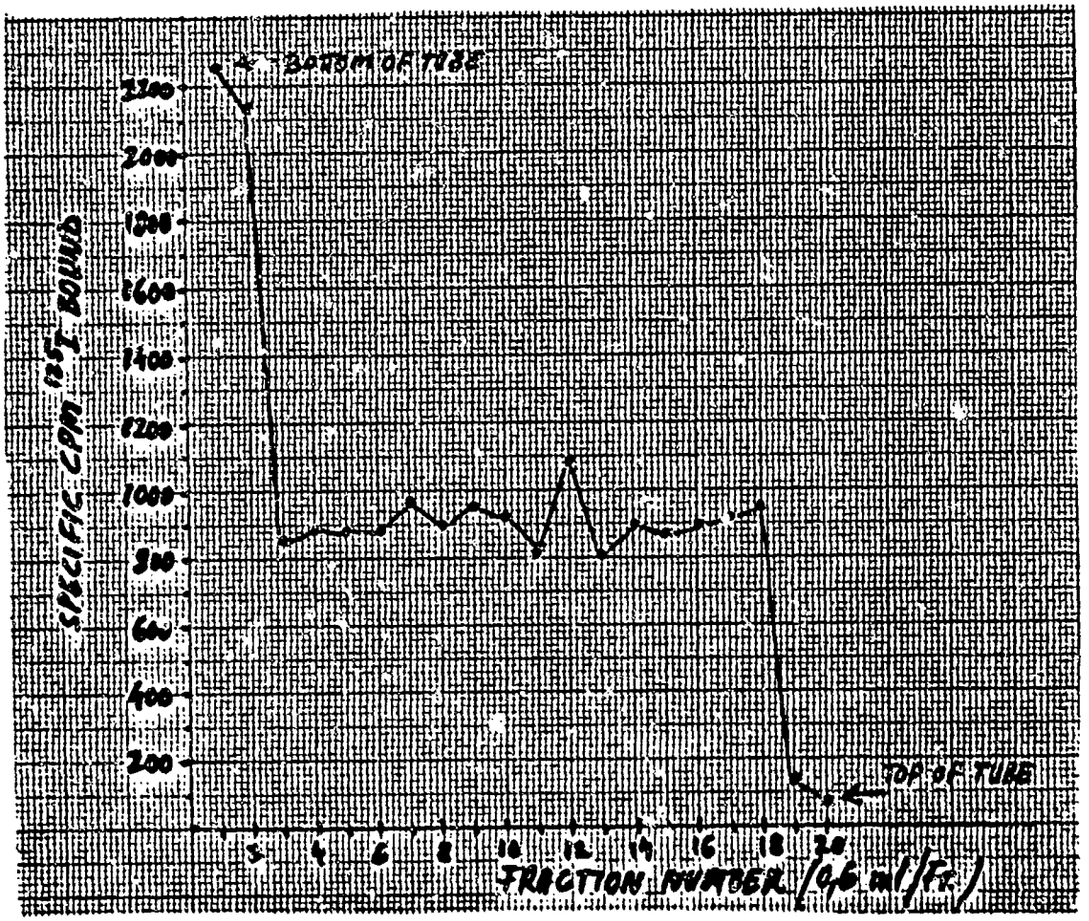


FIGURE 8. Extraction of HAV at pH 7.4 from infected marmoset liver.



Publications

Sanchez, Y., Ionescu-Matiu, I., Dreesman, G.R., Kramp, W., Six, H.R., Hollinger, F.B. and Melnick, J.L. Humoral and cellular immunity to hepatitis B virus-derived antigens: comparative activity of Freund complete adjuvant, alum, and liposomes. Infect. Immun. 30:728-733, 1980.

Dreesman, G.R., Hollinger, F.B., Sanchez, Y., Oefinger, P. and Melnick, J.L. Immunization of chimpanzees with hepatitis B virus-derived polypeptides. Infect. Immun. 32:62-67, 1981.

Sanchez, Y., Ionescu-Matiu, I., Dreesman, G.R., Hollinger, F.B. and Melnick, J.L. Evidence for the presence of repeating antigenic determinants in the major and minor polypeptides derived from hepatitis B surface antigen. Virology, in press.

Benson, J.R., Funk, G.A., Sanchez, Y. and Dreesman, G.R. Evidence for protein homology of the two major hepatitis B surface antigen (HBsAg) derived polypeptides. Submitted for publication.

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